DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.
DMD Fast Forward. Published on November 12, 2009 as doi:10.1124/dmd.109.028407

An In Vitro Assay to Assess Transporter-Based Cholestatic Hepatotoxicity Using Sandwich-Cultured Rat Hepatocytes

John H. Ansede, William R. Smith, Cassandra H. Perry, Robert L. St. Claire III and Kenneth R. Brouwer†

Qualyst, Inc., 2810 Meridian Parkway, Suite 100, Durham, NC, 27713, JHA, WRS, CHP, RLS, and KRB

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Screening for Transporter-Based Cholestatic Hepatotoxicity

Corresponding Author[†]**:** Kenneth R. Brouwer, CSO, Qualyst, Inc., 2810 Meridian Parkway, Suite 100, Durham, NC 27713.

Tel: (919) 313-6502 Fax: (919) 313-0163 Email: KennethBrouwer@Qualyst.com

Number of pages of text: 22

Tables: 0

Figures: 3

References: 21

Words in abstract: 250

Words in introduction: 624

Words in discussion: 1437

Abbreviations: SCH, sandwich-cultured hepatocytes; BEI, biliary excretion index; $Cl_{biliary}$, in vitro biliary clearance

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Drug-induced cholestasis can result from the inhibition of biliary efflux of bile acids in the liver. Drugs may inhibit the hepatic uptake and/or the biliary efflux of bile acids resulting in an increase in serum concentrations. However, it is the intracellular concentration of bile acids that results in hepatotoxicity and thus serum concentrations may not necessarily be an appropriate indicator of hepatotoxicity. In this study, sandwich-cultured rat hepatocytes (SCRH) were used as an *in vitro* model to assess the cholestatic potential of drugs using deuterium labeled sodium taurocholate (d_8-TCA) as a probe for bile acid transport. Eight drugs were tested as putative inhibitors of d_8-TCA uptake and efflux. The hepatobiliary disposition of d_8-TCA in the absence and presence of drugs was measured using LC/MS/MS and the accumulation (hepatocytes and hepatocytes plus bile), biliary excretion index (BEI) and *in vitro* biliary clearance ($Cl_{biliary}$) were reported. Compounds were classified based on inhibition of uptake, efflux or a combination of both processes. Cyclosporine A and glyburide showed a decrease in total (hepatocytes plus bile), an increase in intracellular (hepatocytes only) accumulation, and a decrease in BEI and $Cl_{biliary}$ of d_8-TCA suggesting efflux was primarily affected. Erythromycin-estolate, troglitazone and bosentan resulted in a decrease in accumulation (total and intracellular), BEI and $Cl_{biliary}$ of d_8-TCA suggesting uptake was primarily affected. Determination of a compounds relative effect on bile acid uptake, efflux, and direct determination of alterations in intracellular amounts of bile acids, may provide useful mechanistic information on compounds that cause increases in serum bile acids.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Drug-induced liver toxicity is the single most common reason for withdrawal of FDA-approved drugs from the market (Kaplowitz, 2001). Recent data suggest that hepatic transport proteins may be an important site of toxic interactions, and inhibition of the basolateral uptake and/or canalicular excretion of bile acids (cholestasis) by drugs or metabolites is becoming a well-recognized cause of liver disease (Lewis and Zimmerman, 1999, Kosters and Karpen, 2008). Basolateral transporters are essential in the hepatic uptake of drugs from the blood whereas canalicular transporters are involved in the elimination of drugs and bile acids across the canalicular membrane into the bile. Drugs or metabolites that affect these transporter processes can lead to the intracellular accumulation of bile acids resulting in the development of cholestatic liver damage (Fattinger et al., 2001; Funk et al., 2001b).

Transporters involved in the hepatic uptake of drugs and bile acids from the blood belong to the sodium-dependent and sodium-independent pathways. The sodium taurocholate cotransporting polypeptide (NTCP) accounts for the uptake of 80% of conjugated bile acids (i.e., taurocholic and glycocholic acids) and to a much lesser extent for unconjugated bile acids (Hagenbuch and Dawson, 2004). In addition to NTCP, members of the superfamily of organic anion-transporting polypeptides (OATP) are involved in the sodium-independent transport of bile acids (Hagenbuch and Meier, 2004). Whereas multiple transporters are involved in the hepatic uptake of bile acids, the bile salt export protein (BSEP) is the primary transporter involved in the biliary efflux of conjugated bile acids across the canalicular membrane including taurocholate, glycocholate, chenodeoxycholate, and deoxycholate (Byrne et al., 2002).

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

In addition to their involvement in the transport of bile acids and other endogenous substrates, basolateral and canalicular transport systems are also involved in the transport of drugs. Compounds that compete for similar transport pathways may result in an interaction in which one compound inhibits the transport of another. For example, hepatotoxicity associated with troglitazone and bosentan has been attributed to alterations in hepatic bile acid transport through the inhibition of competing transport pathways (Fattinger et al., 2001; Funk et al., 2001a; Funk et al., 2001b). *In vivo,* bosentan significantly increased serum bile salt levels (Fattinger et al., 2001). Furthermore, *in vitro* results showed BSEP-mediated taurocholate transport was inhibited by bosentan suggesting bosentan-induced liver injury is mediated in part by inhibition of BSEP resulting in intracellular accumulation of bile salts and liver damage.

Most *in vitro* transporter assays using suspended hepatocytes, membrane vesicles or transfected cell lines primarily assess either hepatic uptake or efflux; however, these assays cannot directly evaluate the relative effects of inhibition of hepatic uptake and/or biliary excretion. Because sandwich-cultured hepatocytes (SCH, B-CLEAR®) maintain the expression and function of key uptake and efflux transporters relative to *in vivo*, it is the most relevant system to evaluate and predict the potential of a compound to cause transporter-based liver toxicity. Several reports have described the use of SCH to assess the effect of compounds on the inhibition of bile acid transport; albeit using different methodologies (Kostrubsky et al., 2003; Kemp et al., 2005; Kostrubsky et al., 2006; McRae et al., 2006; Marion et al., 2007). For example, Kostrubsky, used SCH to evaluate the potential of drugs to inhibit bile acid transport (Kostrubsky et al., 2006). However, inhibition of hepatic uptake or biliary efflux could not be distinguished based on the

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

methodology used in their report since biliary efflux may be affected by inhibition of uptake, efflux or a combination of both processes. In this report we describe the development and application of an *in vitro* screen using SCH to evaluate the potential of test compounds to inhibit the transport of deuterium labeled taurocholic acid (d_8-TCA) and define parameters which may be used to differentiate between effects on hepatic uptake and/or biliary efflux.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Materials. Cyclosporine A, erythromycin-estolate, glyburide, nefazodone, , salicylate, troglitazone, and troleandomycin were purchased from Sigma-Aldrich (St. Louis, MO). Bosentan was obtained from Toronto Research Chemicals (North York, Ontario, Canada). Stock solutions were prepared at 5 and 50 mM in 100% DMSO (troglitazone was prepared at 1 and 10 mM) and stored at -20°C. The sodium salt of the stable isotope, d8-taurocholic acid (Ethanesulfonic acid, 1,1,2,2-tetradeutero-2-[[(3α,5β,7α,12α)-2,2,4,4-tetradeutero-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]-, monosodium salt), (d8-TCA) was synthesized by Martrex, Inc. (Minnetonka, MN). The internal standard, d4-taurocholic acid (d4-TCA), (catalog # T008850), was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). HPLC grade methanol from Fisher Scientific (Fair Lawn, NJ) and Fluka Mass Spectrometry grade ammonium acetate from Sigma-Aldrich (Milwaukee, WI) were utilized for sample preparation and analysis.

Isolation, Plating and Maintenance of Sandwich-Cultured Rat Hepatocytes. Hepatocytes were isolated from male Wistar rats (250-300 g) using a two-step, single path re-circulating collagenase perfusion as reported previously (LeCluyse et al., 1996). Cells were suspended at ca. 1 x 10^6 cells/mL in medium and subsequently added at a volume of approximately 1.5 mL per well to 6-well BIOCOAT® plates (BD Biosciences, Bedford, MA). Post-plating (1 - 3 hours), non-adherent cells were removed by aspiration and replaced with fresh plating medium. Following 24 hours of incubation, cells were overlaid with 2 mL of 0.25 mg/mL Matrigel™ (BD Biosciences) solution prepared in

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

culture medium. Culture medium was replaced every 24 hours and uptake studies were conducted on day-4 of culture.

Hepatobiliary disposition of d_8-taurocholic acid (d_8-TCA) in SCRH. Hepatocytes were washed three times with one milliliter of either Hank's Balanced Salt Solution containing calcium (standard buffer) or Hank's Balanced Salt Solution without calcium containing 0.38 g/L EGTA (calcium-free buffer) and incubated with third wash either in the presence or absence of test compound (5 and 50 µM, except for troglitazone, which was evaluated at 1 and 10 µM) for 10 minutes at 37°C. Incubation in standard buffer maintains the integrity of the tight junctions, while incubation in calcium-free buffer opens the tight junctions. Following the initial incubation, the hepatocytes were washed, and d_8-TCA (2.5 µM) and test compound were added to the hepatocytes and incubated. Following a 10 minute co-incubation, the hepatocytes were washed and frozen at -80ºC for later analysis., of d_8-TCA. d_8-TCA, measured by LC/MS/MS analysis as described below, was used to distinguish between the probe and the endogenous taurocholate produced in sandwich-cultured hepatocytes. Total protein per well was determined from separate plates from the same lot of hepatocytes using a BCA™ protein assay kit (Thermo Scientific, Rockford, IL) and d_8-TCA mass (pmol) was normalized to protein content for each well. The amount of d_8-TCA excreted into the bile pockets was determined by subtracting the amount of d_8-TCA in the lysates from cells exposed to calcium-free buffer (hepatocytes) from the amount of d_8-TCA in the lysates from cells exposed to standard buffer (hepatocytes + bile pockets).

Kinetic studies were performed in SCH using the same protocol as described above in the absence of test compound. The hepatobiliary disposition of d_8-TCA was

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

evaluated over a concentration range of 0.1 to 50 µM and an incubation time of 1 to 20 minutes. Stock concentrations of d_8-TCA were prepared such that the final DMSO concentrations did not exceed 0.15%.

Sample Preparation for LC/MS/MS analysis. A volume of 750 µL of lysis solution, [70:30 methanol:water (v:v) containing 25 nM d_4-TCA (internal standard)], was added to each well of previously frozen 6-well plates containing study samples or standards. Plates were shaken for approximately 15 min and the cell lysate solution was transferred to a Whatman® 96-well Unifilter® Plate (Whatman, Florham Park, NJ). Lysate was filtered into a deepwell plate by centrifugation (2000 g for 5 min). The sample filtrate was evaporated to dryness and the samples were reconstituted in 300 µL sample diluent containing 60% methanol and 40% 10mM ammonium acetate (native pH), and mixed for 15 min on a plate shaker. The reconstituted samples were transferred to a Whatman® 96-well Unifilter® Plate and filtered into a Costar 3956 plate by centrifugation (2000 g for 5 min) and sealed with a silicone capmat prior to LC/MS/MS analysis.

LC/MS/MS Analysis. A Shimadzu binary HPLC system (Columbia, MD) composed of LC-10ADvp pumps, a CTO-10Avp oven, and an HTc – 96-well autosampler were used. The chromatographic column was either a Thermo Scientific (Bellefont, PA) BetaBasic™-18 (100 x 1.0 mm, 3 µm) or a Hypersil Gold C18 (100 x 1.0 mm, 3 µm). Column temperature was maintained at 35°C. A mobile phase gradient composed of 0.5 mM ammonium acetate (native pH) and methanol was used at a flow rate of 50 µL/min and a total run time of 10 min. The d_8-TCA retention time was approximately 5 min. An injection volume of 10 µL was used. The kinetic studies used an injection volume of 1 µL for the analysis of samples containing 100 – 1000 pmol/well of analyte. Tandem mass

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

spectrometry with negative ion electrospray ionization was conducted with a Thermo Electron TSQ® Quantum Discovery MAX™ (Waltham, MA) with an Ion Max ESI source. The transitions monitored at unit resolution for d_8-TCA and d_4-TCA (internal standard) were (precursor m/z > product m/z) 522 > 128 and 518 > 124, respectively.

Data Analysis. Accumulation was calculated in hepatocytes plus bile (standard buffer) and hepatocytes (calcium-free buffer) by subtracting the amount of d_8-TCA (expressed as pmol/well) from control plates (non-specific binding) from the amount of d_8-TCA (expressed as pmol/well) and dividing by protein content (expressed as mg protein/well). The biliary excretion index (BEI) was calculated according to the following equation:

$$BEI = \frac{Accumulation_{S\tan dard} - Accumulation_{Calcium-free}}{Accumulation_{S\tan dard}} \times 100$$

The *in vitro* biliary clearance ($Cl_{biliary}$) was determined using the following equation, and was scaled to body weight using 0.2 g protein/g liver weight, and 40 g liver/kg body weight (Seglen, 1976):

$$Cl_{biliary} = \frac{Accumulation_{Standard} - Accumulation_{Calcium-free}}{AUC\ \ (i.e.\, Time \bullet Concentration_{Media})}$$

The accumulation (standard and calcium free buffer), BEI, and *in vitro* biliary clearance for d_8-TCA was expressed as a percent of the control value (no test compound). Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparison test. A P value ≤ 0.05 was considered significant.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

LC/MS/MS analysis. The uptake and biliary efflux of d_8-TCA in SCRH was quantitated using LC/MS/MS analysis.

Kinetics of d_8-TCA Uptake and Efflux in SCRH. The effect of substrate concentration and incubation time on the hepatobiliary disposition of d_8-TCA was determined in SCRH and the results are presented in Figure 1 (A and B). The results from time-dependent accumulation of d_8-TCA in hepatocytes or hepatocytes plus bile pockets are presented in Figure 1A. A concentration of 2.5 µM d_8-TCA was chosen since it represented a concentration within a linear range of accumulation. The accumulation of d_8-TCA in hepatocytes plus bile pockets was linear over the 20 minute time period evaluated.

An increase in d_8-TCA concentration resulted in an increase in intracellular accumulation (Figure 1B), i.e. accumulation in calcium-free buffer representing the mass of d_8-TCA accumulated in hepatocytes only. Saturable uptake of d_8-TC was observed within the concentration range evaluated. A concentration-dependent increase in d_8-TCA accumulation was observed in standard buffer incubations representing the mass of d_8-TCA accumulated in hepatocytes plus bile pockets (Figure 1B). Accumulation of d_8-TCA in hepatocytes plus bile pockets reached a maximum value at 25 µM d_8-TCA. Kinetic constants (K_m and V_{max}) were not determined since accumulation in hepatocytes or hepatocytes plus bile represents several processes including; uptake into hepatocytes, efflux across the basolateral membrane and efflux across the canalicular membrane into bile.

The Effect of Test Compounds on the Hepatobiliary Disposition of d_8-TCA in SCRH. The effects of test compounds (all 50 µM, except troglitazone - 10 µM) on the

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

accumulation, biliary excretion index (BEI), and *in vitro* biliary clearance ($Cl_{biliary}$) of d_8-TCA were evaluated in SCRH and the results are presented as % control (no test compound) in Figures 2 and 3.

In the absence of test compound, the accumulation of d_8-TCA in incubations with standard buffer (hepatocytes and bile pockets) and calcium-free buffer (hepatocytes only) was 131 ± 31.4 and 15.1 ± 2.47 pmol/mg protein (mean ± standard error), respectively. Cyclosporine and glyburide significantly decreased the accumulation of d_8-TCA in standard buffer incubations; however, both compounds showed a significant increase in the accumulation of d_8-TCA in calcium-free buffer incubations as presented in Figure 2. Erythromycin-estolate, troglitazone and bosentan significantly decreased the accumulation of d_8-TCA in both standard and calcium-free buffer incubations. Nefazodone significantly decreased the accumulation of d_8-TCA in standard buffer incubations and had no effect on the accumulation of d_8-TCA in calcium-free buffer incubations. Salicylate and troleandomycin had no effect on the accumulation of d_8-TCA in either incubation.

The effects of test compounds on the biliary efflux of d_8-TCA were measured and presented as BEI and $Cl_{biliary}$ (Figure 3). In the absence of test compound, the BEI for d_8-TCA was 88.3 ± 1.32 %, indicating that 88 % of the d_8-TCA taken up by the hepatocytes was effluxed into the bile. Of the eight compounds evaluated, glyburide and cyclosporine A had the greatest inhibitory effect on the BEI (Figure 3), reducing the BEI of d_8-TCA to 21 and 34% of control, respectively. Erythromycin-estolate, nefazodone, bosentan and troglitazone showed an ca. 20 – 35% inhibitory effect on the BEI of d_8-TCA. The

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

decreases in BEI were all statistically significant, except for salicylate and troleandomycin (TAO) which had no effect on the BEI of d_8-TCA.

The $Cl_{biliary}$ of d_8-TCA in the absence of inhibitor was 37.1 ± 9.54 mL/min/kg. All test compounds evaluated, with the exception of salicylate and TAO, showed a statistically significant decrease in the *in vitro* biliary clearance of d_8-TCA.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Cholestasis can be defined as any condition in which substances normally excreted into bile are retained. The most common method for the clinical determination of cholestasis is to measure serum concentrations of bile acids or conjugated bilirubin. Bile acids are strong detergents that cause cell membrane injury and impairment of membrane function. However, an increase in serum bile acid concentrations may not necessarily reflect an increase in intracellular hepatocyte concentrations. It is generally assumed that it is the concentration of bile acids inside the hepatocyte that is the primary determinate of hepatotoxicity. Thus, it is important to differentiate between a compound's effect on the uptake or efflux of bile acids, since inhibition of uptake will result in decreased hepatocellular concentrations of bile acids which would be less likely to cause hepatotoxicity, whereas, inhibition of efflux (either basolateral and/or canalicular) would result in increased hepatocyte concentrations of bile acids, increasing the potential for hepatotoxicity. Inhibition at either site will result in increased serum concentration of bile acids and therefore increased serum levels may not necessarily reflect the hepatotoxic potential of a drug.

Transporter-based drug interactions that inhibit basolateral uptake or efflux (basolateral or canalicular) in the liver may lead to the alteration of the *in vivo* hepatobiliary disposition of bile acids (Fattinger et al., 2001; Funk et al., 2001a; Funk et al., 2001b; Pauli-Magnus and Meier, 2006). Knowledge of the site of inhibition is important in understanding the relationship between elevated serum concentrations of bile acids and the intracellular concentration of bile acids in hepatocytes leading to hepatotoxicity. Unlike other *in vitro* transporter models, SCH can simultaneously

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

determine the potential for a compound to alter the basolateral uptake and/or the basolateral or canalicular efflux of bile acids and therefore predict the overall effect a drug may have on bile acid disposition *in vivo* (Kemp et al., 2005). Inhibition of basolateral efflux of bile acids, could result in an increase in the intracellular concentration of bile acids. If the increased levels of bile acids are within the linear range of the canalicular efflux transporters, the BEI should not change, however the *in vitro* biliary clearance could increase under this condition.

Treatment with cyclosporine A, glyburide, erythromycin-estolate, troglitazone and bosentan decreased the *in vitro* biliary clearance of d_8-TCA to less than 20 % of the control value. The *in vitro* biliary clearance is an indicator of the overall effect of the compound on bile acid excretion. A decrease in the *in vitro* biliary clearance reflects a decrease in the amount of d_8-TCA excreted into the bile, which can result from inhibition of either; (i) basolateral uptake transporters, or (ii) canalicular efflux transporters. The biliary excretion index (BEI) represents the fraction of the total mass of d_8-TCA taken up that is excreted into the bile. A decrease in the BEI represents inhibition of d_8-TCA efflux into the bile. The BEI, in conjunction with the accumulation and *in vitro* biliary clearance, can be used to determine the site of action (basolateral/uptake versus canalicular/efflux) of a particular compound on the hepatobiliary disposition of bile acids in SCH.

Cyclosporine and glyburide resulted in a decrease in the accumulation of d_8-TCA in samples treated with standard buffer representing a decrease in the total mass of d_8-TCA accumulated in hepatocytes plus bile pockets. Both compounds also decreased the BEI of taurocholate to 34 and 21 % of control, suggesting an inhibition of bile acid efflux

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

out of the hepatocyte. Both compoundsincreased the mass of d_8-TCA in calcium-free treated samples (representing an increase in hepatocellular concentration). This suggests that canalicular (and/or basolateral) efflux processes were inhibited to a greater extent than uptake processes, resulting in an increase in the hepatocellular concentration of bile acids. The potential for cyclosporine A and glyburide to increase the levels of bile acids inside of the hepatocytes may be related to their potential for *in vivo* hepatotoxicity. These observations are consistent with previous reports showing increased bile acid levels in the livers of rats treated with cyclosporine A and glyburide resulting in cholestatic hepatotoxicity (Chan and Shaffer, 1997; Mizuta et al., 1999; Kostrubsky et al., 2003). Hepatotoxicity was also reported in human subjects treated with cyclosporine A resulting in a 2- to 3-fold increase in total serum bile acids (Kassianides, 1990, Tripodi et al., 2002), which is consistent with the *in vitro* results obtained in this study using SCRH. However, cyclosporine A and glyburide are not associated with high incidences of hepatotoxicity *in vivo* with only a few cases of cholestatic hepatotoxicity being reported with these drugs. The low incidences of hepatotoxicity associated with these drugs could be explained by the low *in vivo* plasma concentrations of these drugs (0.2 µM glyburide and 8.3 – 332 nM cyclosporine A), or differential intracellular accumulation in human hepatocytes due to differences in kinetic properties of uptake and efflux transporters between rat and human hepatocytes.

Erythromycin-estolate, an erythromycin analogue used in the treatment of bacterial infections has been associated with cholestatic liver injury. In SCRH, erythromycin-estolate decreased d_8-TCA accumulation by ca. 70 and 90% in hepatocytes and hepatocytes plus bile, respectively. This resulted in a decrease in the $Cl_{biliary}$ of d_8-

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

TCA to less than 5% of control. However, the BEI decreased to only 63% of control, suggesting that erythromycin-estolate has more of an effect on the uptake of d_8-TCA into the hepatocyte than on the efflux of d_8-TCA into the bile. Bosentan and troglitazone demonstrated similar effects on the hepatobiliary disposition of d_8-TCA in SCRH as observed for erythromycin-estolate. The $Cl_{biliary}$ of d_8-TCA decreased to ca. 15% of control for bosentan and troglitazone. d_8-TCA accumulation decreased by ca. 50 and 70% in hepatocytes and hepatocytes plus bile, respectively, whereas the BEI decreased by less than 25% suggesting that troglitazone and bosentan have a potent affect on uptake of d_8-TCA in addition to their inhibition of the efflux of d_8-TCA into bile. These results are consistent with the findings of Leslie et al. in which bosentan was identified as a potent inhibitor of rat Ntcp (Leslie et al., 2007). Furthermore, Kemp et al. also observed inhibition of hepatic uptake and biliary efflux of TCA by troglitazone co-administration in SCRH (Kemp et al., 2005). At the concentrations evaluated in our experiments, troglitazone had a greater effect on hepatic uptake than on biliary efflux as accumulation was inhibited to a greater extent than the BEI. In vivo studies in rats demonstratedthat troglitazone and its metabolites inhibited the efflux of bile acids by interfering with the canalicular efflux transporter, Bsep (Funk et al., 2001a; Funk et al., 2001b).

Nefazodone had no effect on the accumulation of d_8-TCA in hepatocytes; however, resulted in an ca. 50% decrease in accumulation in hepatocytes plus bile (consistent with an effect on uptake). The lack of an effect on the accumulation of d_8-TCA in hepatocytes along with the decrease in the accumulation in hepatocytes plus bile and the decrease in the BEI indicates that nefazodone is inhibiting both uptake and efflux of d_8-TCA. If inhibition were strictly on the efflux processes then the accumulation in

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

hepatocytes would be greater than that of control as seen for cyclosporine A and glyburide. However, accumulation in hepatocytes was similar to control values so in order to decrease the mass excreted in bile both hepatic uptake and efflux must be inhibited to some extent. Nefazodone has been reported to increase serum bile acids in rat (Kostrubsky et al., 2006) and was withdrawn from the market due to hepatotoxicity (Spigset et al., 2003).

Salicylate, and troleandomycin were used as negative controls to demonstrate that compounds with no reported *in vivo* cholestatic potential have no effect on uptake and/or efflux of d_8-TCA in SCRH.

The observations of increased hepatocellular amounts of bile acids in the presence of cyclosporine A and glyburide indicate that it is important to utilize methodology that can simultaneously evaluate the effect of inhibitor compounds on the uptake and efflux of bile acids. Determination of the hepatocellular amount of bile acids allows for the differentiation between compounds that cause a decrease in the *in vitro* biliary clearance, resulting from inhibition of uptake and/or efflux of bile acids, and those that cause a decrease in the *in vitro* biliary clearance by strongly inhibiting the efflux of bile acids into the bile. Methodologies that evaluate only efflux, cannot quantitate the role of uptake transporter inhibition relative to the inhibition of bile acid efflux (Kostrubsky et al., 2006). B-CLEAR® sandwich-cultured hepatocytes have also demonstrated the capacity to synthesize endogenous bile acids. Following four days of culture, taurocholic acid, glycocholic acid, and the taurine and glycine conjugates of chenodeoxycholic acid, and muricholic acid have been detected inside the hepatocytes using LCMS analysis. The effect of drugs on the production and hepatobiliary disposition of endogenous bile acids

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

may offer additional insights to the hepatotoxic effects of drugs, and is an area of ongoing research in our labs.

In summary, SCH may serve as an *in vitro* model to assess the cholestatic potential of drug candidates. The methodology used in this study allows for the simultaneous assessment of both hepatic uptake and biliary efflux and assessment of alterations in the hepatocellular amounts of bile acids. The site of inhibition may be an important parameter in understanding whether increased serum bile acids *in vivo* may lead to cholestatic hepatotoxicity (inhibition of efflux leading to an increase in intracellular bile acids). Furthermore, a deuterium-labeled taurocholate analogue (d_8-TCA) may serve as a useful probe for assessing hepatobiliary disposition of bile acids in SCH eliminating the need for use of radiolabeled probes.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Byrne JA, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ and Thompson RJ (2002) The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* 123:1649-1658.

Chan FK and Shaffer EA (1997) Cholestatic effects of cyclosporine in the rat. *Transplantation* 63:1574-1578.

Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B and Meier PJ (2001) The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther* 69:223-231.

Funk C, Pantze M, Jehle L, Ponelle C, Scheuermann G, Lazendic M and Gasser R (2001a) Troglitazone-induced intrahepatic cholestasis by an interference with the hepatobiliary export of bile acids in male and female rats. Correlation with the gender difference in troglitazone sulfate formation and the inhibition of the canalicular bile salt export pump (Bsep) by troglitazone and troglitazone sulfate. *Toxicology* 167:83-98.

Funk C, Ponelle C, Scheuermann G and Pantze M (2001b) Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat. *Mol Pharmacol* 59:627-635.

Hagenbuch B and Dawson P (2004) The sodium bile salt cotransport family SLC10. *Pflugers Arch* 447:566-570.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* 447:653-665.

Kassianides, C, Nussenblatt, R, Palestine, A.G., Mellow, S.D., and Hoofnagle, J.H. (1990) Liver injury from Cyclosporine A. Digestive Diseases and Sciences, 35, 6: 693-697.

Kaplowitz N (2001) Drug-induced liver disorders: implications for drug development and regulation. *Drug Saf* 24:483-490.

Kemp DC, Zamek-Gliszczynski MJ and Brouwer KL (2005) Xenobiotics inhibit hepatic uptake and biliary excretion of taurocholate in rat hepatocytes. *Toxicol Sci* 83:207-214.

Kosters A and Karpen SJ (2008) Bile acid transporters in health and disease. *Xenobiotica* 38:1043-1071.

Kostrubsky SE, Strom SC, Kalgutkar AS, Kulkarni S, Atherton J, Mireles R, Feng B, Kubik R, Hanson J, Urda E and Mutlib AE (2006) Inhibition of hepatobiliary transport as a predictive method for clinical hepatotoxicity of nefazodone. *Toxicol Sci* 90:451-459.

Kostrubsky VE, Strom SC, Hanson J, Urda E, Rose K, Burliegh J, Zocharski P, Cai H, Sinclair JF and Sahi J (2003) Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. *Toxicol Sci* 76:220-228.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

LeCluyse EL, Bullock P, Parkinson A and Hochman JH (1996) Cultured rat hepatocytes., in *Model Systems for Biopharmaceutical Assessment of Drug Absorption and Metabolism.* (Borchardt RT, Wilson G and Smith P eds), Plenum, New York.

Leslie EM, Watkins PB, Kim RB and Brouwer KL (2007) Differential inhibition of rat and human Na+-dependent taurocholate cotransporting polypeptide (NTCP/SLC10A1)by bosentan: a mechanism for species differences in hepatotoxicity. *J Pharmacol Exp Ther* 321:1170-1178.

Lewis J., Zimmerman H. (1999) Drug- and chemical- induced cholestasis. *Clin. Liver Disease* 3,3:433-464.

Marion TL, Leslie EM and Brouwer KL (2007) Use of sandwich-cultured hepatocytes to evaluate impaired bile acid transport as a mechanism of drug-induced hepatotoxicity. *Mol Pharm* 4:911-918.

McRae MP, Lowe CM, Tian X, Bourdet DL, Ho RH, Leake BF, Kim RB, Brouwer KL and Kashuba AD (2006) Ritonavir, saquinavir, and efavirenz, but not nevirapine, inhibit bile acid transport in human and rat hepatocytes. *J Pharmacol Exp Ther* 318:1068-1075.

Mizuta K, Kobayashi E, Uchida H, Ogino Y, Fujimura A, Kawarasaki H and Hashizume K (1999) Cyclosporine inhibits transport of bile acid in rats: comparison of bile acid composition between liver and bile. *Transplant Proc* 31:2755-2756.

Pauli-Magnus C and Meier PJ (2006) Hepatobiliary transporters and drug-induced cholestasis. *Hepatology* 44:778-787.

Seglen PO (1976) Preparation of isolated rat liver cells. *Methods Cell Biol* 13:29-83.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

Spigset O, Hagg S and Bate A (2003) Hepatic injury and pancreatitis during treatment with serotonin reuptake inhibitors: data from the World Health Organization (WHO) database of adverse drug reactions. *Int Clin Psychopharmacol* 18:157-161.

Tripodi V, Nunez M, Carducci C, Mamianetti A and Agost Carreno C (2002) Total serum bile acids in renal transplanted patients receiving cyclosporine A. *Clin Nephrol* 58:350-355.

DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

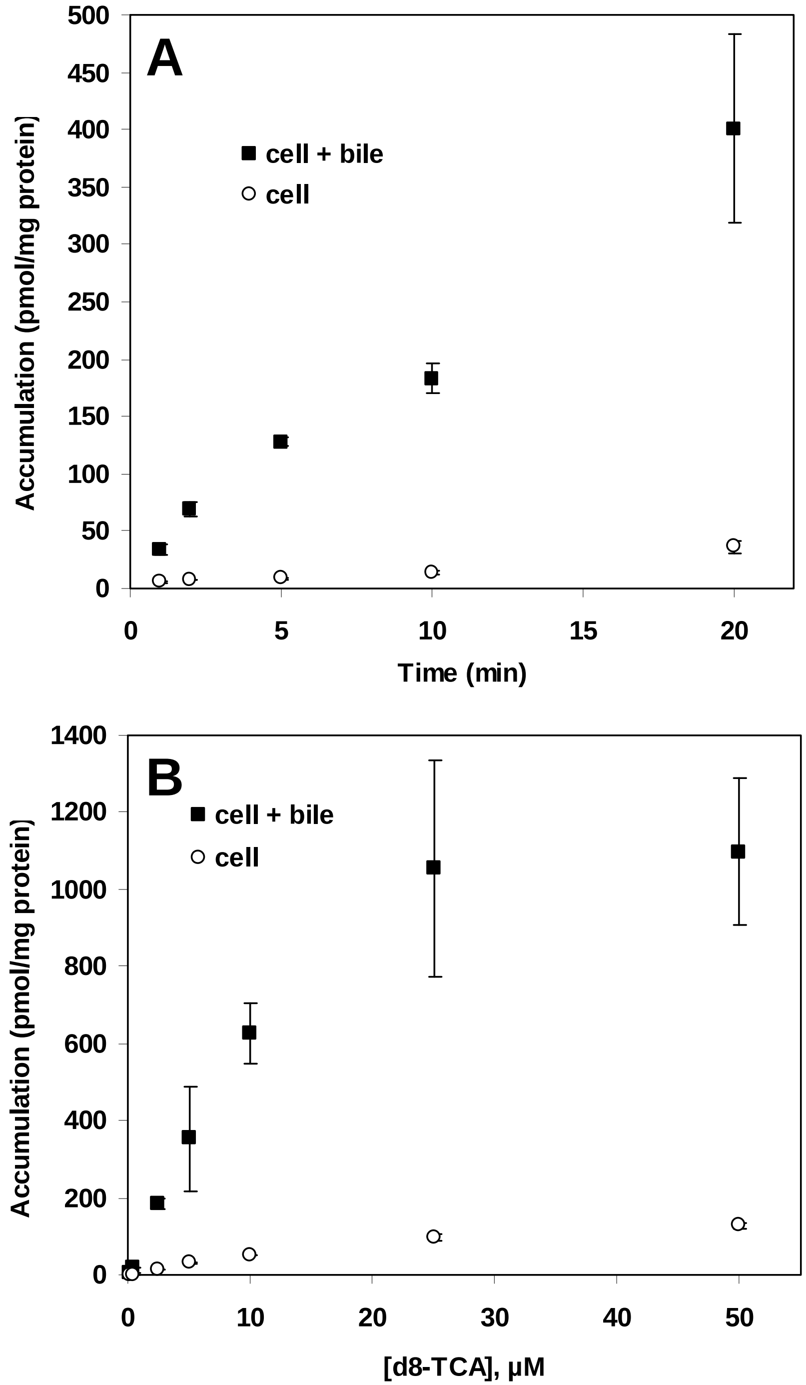
LEGENDS FOR FIGURES

Figure 1. (A) Time (d_8-TCA at 2.5 µM) and (B) Concentration-dependent (10 minute incubation time) accumulation of d_8-TCA in SCRH in incubations treated with standard buffer (hepatocytes plus bile) and calcium-free buffer (hepatocytes only).

Figure 2. The effect of test compounds (50 µM) on the accumulation of d_8-TCA (2.5 µM) in SCRH in incubations treated with standard buffer (hepatocytes plus bile) and calcium-free buffer (hepatocytes only). Data is expressed as mean ± standard error (n=3) of % control (no Test Compound). * indicates a statistically significant difference from control, $P \leq 0.05$.

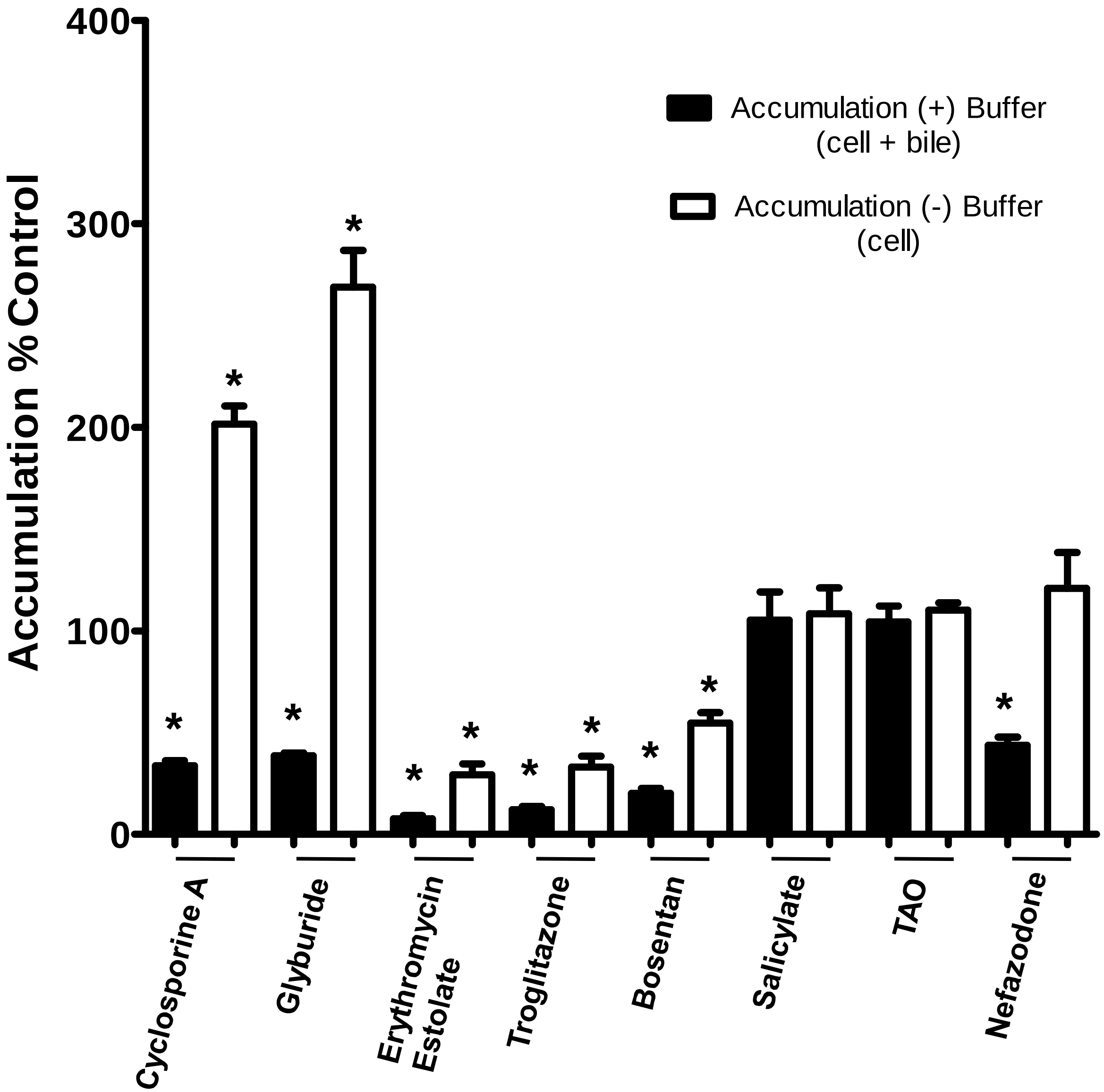
Figure 3. The effect of test compounds (50 µM) on the BEI and $Cl_{biliary}$ of d_8-TCA (2.5 µM) in SCRH. Data is expressed as mean ± standard error (n=3) of % control (no Test Compound). * indicates a statistically significant difference from control, $P \leq 0.05$.

Figure 1



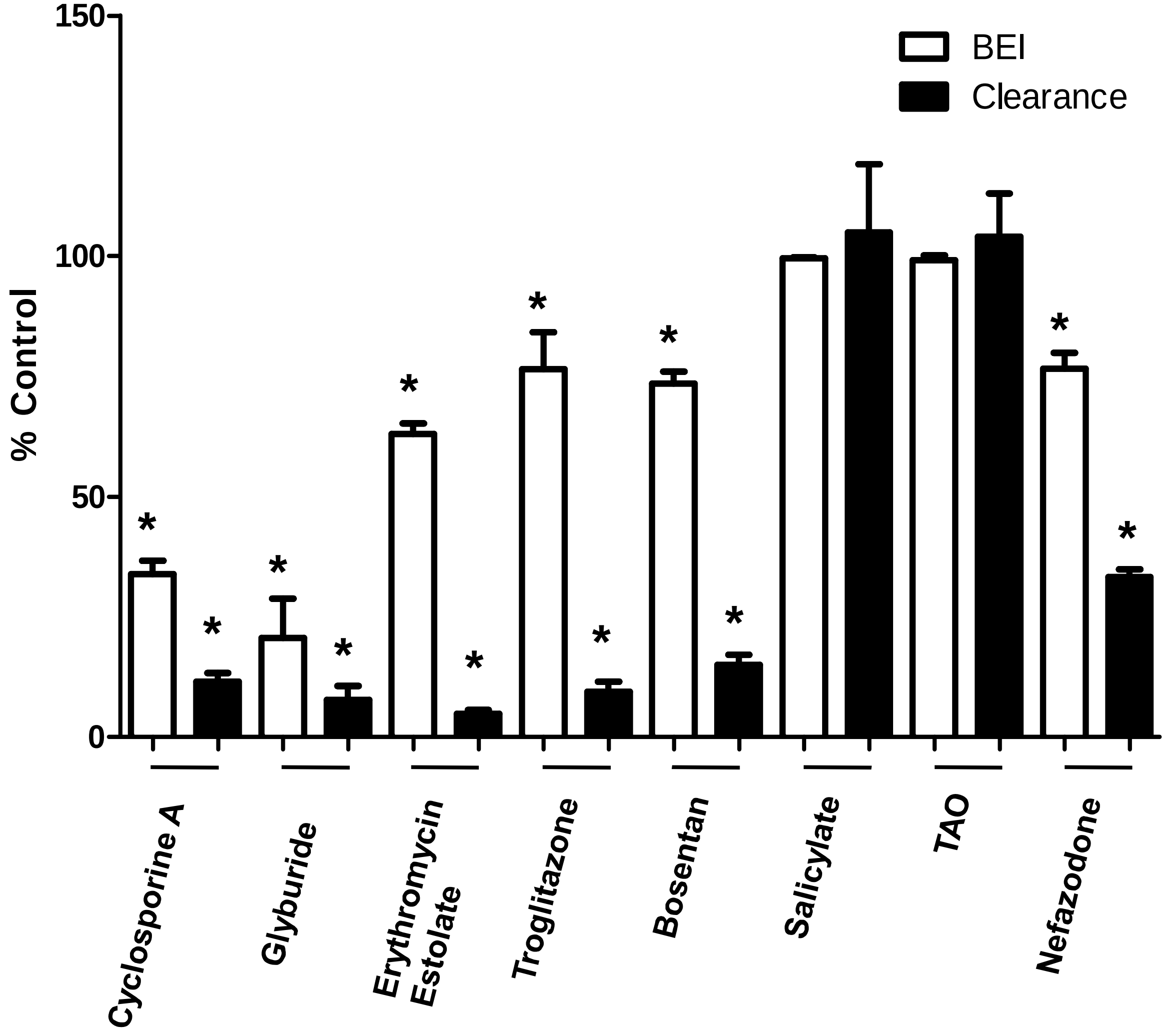
DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



DMD Fast Forward. Published on November 12, 2009 as DOI: 10.1124/dmd.109.028407
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



150
100
50
0
% Control
BEI
Clearance
Cyclosporine A
Glyburide
Erythromycin Estolate
Troglitazone
Bosentan
Salicylate
TAO
Nefazodone